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Iron-Dependent Hydrogenases of *Entamoeba histolytica* and *Giardia lamblia:* Activity of the Recombinant Entamoebic Enzyme and Evidence for Lateral Gene Transfer

JULIE E. J. NIXON[1], JESSICA FIELD[1], ANDREW G. McARTHUR[2], MITCHELL L. SOGIN[2], NIGEL YARLETT[3], BRENDAN J. LOFTUS[4], AND JOHN SAMUELSON[1,*]

[1] *Department of Immunology and Infectious Diseases, Harvard School of Public Health, 665 Huntington Ave., Boston, Massachusetts;* [2] *Josephine Bay Paul Center for Comparative Molecular Biology and Evolution, Marine Biological Laboratory, Woods Hole, Massachusetts;* [3] *Department of Biochemistry, Pace University, New York, New York; and* [4] *The Institute for Genomic Research, Rockville, Maryland*

Abstract. *Entamoeba histolytica* and *Spironucleus barkhanus* have genes that encode short iron-dependent hydrogenases (Fe-hydrogenases), even though these protists lack hydrogenosomes. To understand better the biochemistry of the protist Fe-hydrogenases, we prepared a recombinant *E. histolytica* short Fe-hydrogenase and measured its activity *in vitro*. A *Giardia lamblia* gene encoding a short Fe-hydrogenase was identified from shotgun genomic sequences, and RT-PCR showed that cultured entamoebas and giardias transcribe short Fe-hydrogenase mRNAs. A second *E. histolytica* gene, which encoded a long Fe-hydrogenase, was identified from shotgun genomic sequences. Phylogenetic analyses suggested that the short Fe-hydrogenase genes of entamoeba and diplomonads share a common ancestor, while the long Fe-hydrogenase gene of entamoeba appears to have been laterally transferred from a bacterium. These results are discussed in the context of competing ideas for the origins of genes encoding fermentation enzymes of these protists.

Introduction

One of the great recent discoveries in the cell biology of protists is that the hydrogenosome of *Trichomonas vaginalis*, cause of vaginitis, is a modified mitochondrion, in which the enzymes of oxidative phosphorylation have been replaced by fermentation enzymes that produce hydrogen gas (Müller, 1993, 1998; Bui *et al.*, 1996; Horner *et al.*, 1996; Andersson and Kurland, 1999; Rotte *et al.*, 2000). Proof of this idea includes the presence of mitochondrion-like chaperones and a mitochondrion-like ATP/ADP transporter within the hydrogenosome, as well as organelle-targeting sequences at the N-termini of hydrogenosomal proteins that are encoded in the nucleus (Johnson *et al.*, 1990; Hrdy and Müller, 1995a, b; Bui *et al.*, 1996; Bui and Johnson, 1996; Horner *et al.*, 1996; Bradley *et al.*, 1997; Dyall *et al.*, 2000).

The common origin of hydrogenosomes and mitochondria is included in a new biochemical explanation for the origin of mitochondria, called the hydrogen hypothesis (Martin and Müller, 1998). The hydrogen hypothesis, a revision of the widely accepted endosymbiont hypothesis (Gray *et al.*, 1999), suggests that the α-proteobacterium, which became the mitochondrion, was a facultative anaerobe that was selected for its ability to produce hydrogen in a methanogenic archaeal host. Consistent with this idea, multiple hydrogenosomal fermentation enzymes—including ferredoxin, succinyl-CoA synthetase, and malic enzyme—resemble their counterparts in mitochondria (Johnson *et al.*, 1990; Hrdy and Müller, 1995b). One alternative hypothesis suggests that the mitochondrial endosymbiont was selected for its ability to consume oxygen and thus protect the proto-eukaryote from oxidative damage (Andersson and Kurland, 1999). Another alternative hy-

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* To whom correspondence should be addressed. Current address: Department of Molecular and Cell Biology, Boston University Goldman School of Dental Medicine, 715 Albany St., Boston, MA 02118. E-mail: jsamuels@bu.edu

pothesis suggests that the endosymbiosis was based upon cycling of sulfur (Searcy, 1992).

An iron-dependent hydrogenase (Fe-hydrogenase), which gives the hydrogenosome its name, transfers electrons from reduced ferredoxin to two protons to make hydrogen gas (Lindmark and Müller, 1973; Müller, 1993; Payne *et al.*, 1993; Bui and Johnson, 1996; Horner *et al.*, 2000). Fe-hydrogenase activity, which is detected using hydrogen gas and methyl viologen as a reporter, has been shown in extracts of eukaryotes with hydrogenosomes or plastids (Payne *et al.*, 1993; Wunschiers *et al.*, 2001). Fe-hydrogenases, which are also present in strictly anaerobic bacteria, are designated as long or short depending upon the number of N-half ferredoxin-like domains that are adjacent to a conserved C-half hydrogenase domain (Cammark, 1992). The structures of a short Fe-hydrogenase of *Desulfovibrio desulfuricans* and a long Fe-hydrogenase of *Clostridium pasteurianum* have been solved, and each revealed two ferredoxin-like [4Fe-4S] iron-sulfur centers and a hydrogenase active-site composed of a [4Fe-4S] center bridged to a [2Fe] cluster (Peters *et al.*, 1998; Nicolet *et al.*, 1999).

The reduced ferredoxin is produced within the hydrogenosome by pyruvate:ferredoxin oxidoreductase (PFOR), which decarboxylates pyruvate to acetyl-CoA and CO_2 (Lindmark and Müller, 1973; Müller, 1993; Payne *et al.*, 1993; Hrdy and Müller, 1995a; Horner *et al.*, 1999). It has been difficult to determine the phylogeny of the genes that encode Fe-hydrogenase and PFOR, because these iron-sulfur proteins are absent from mitochondria, α-proteobacteria, and most other eubacteria. The *pfor* genes of *T. vaginalis*, *Entamoeba histolytica* (cause of amoebic dysentery), and *Giardia lamblia* (a cause of diarrhea) appear to share a common ancestry, although no bacterial donor has been identified, and a gene encoding a second keto-acid oxidoreductase of *G. lamblia* appears to have a distinct ancestry (Reeves, 1984; Hrdy and Müller, 1995a; Rosenthal *et al.*, 1997; Brown *et al.*, 1998; Müller, 1998; Horner *et al.*, 1999; Huston and Petri, 2001). The *fe-hydrogenase* genes of *T. vaginalis*, *E. histolytica*, and *Spironucleus barkhanus* (a diplomonad similar to *G. lamblia*), each of which encodes a short Fe-hydrogenase, also appear to share a common ancestry, although no bacterial donor was identified (Horner *et al.*, 2000; van Hoek *et al.*, 2000). In contrast, the long *fe-hydrogenase* gene of the microaerophilic ciliate *Nyctotherus ovalis* appears to have a distinct ancestry.

The Fe-hydrogenase results are surprising for three reasons (Horner *et al.*, 2000). First, the fermentation enzymes of *E. histolytica* and *G. lamblia* are present in the cytosol rather than in an organelle (Reeves, 1984; Müller, 1993; Brown *et al.*, 1998; Mai *et al.*, 1999; Ghosh *et al.*, 2000). Entamoebas have a mitochondrion-derived organelle called the crypton or mitosome, while the giardial gene encoding a 60-kDa chaperonin appears to be endosymbiont-derived, but no organelle has been identified (Clark and Roger, 1995; Roger *et al.*, 1998; Mai *et al.*, 1999; Tovar *et al.*, 1999; Ghosh *et al.*, 2000). Second, entamoebas and diplomonads have long been thought not to produce hydrogen gas in culture, although a recent report suggests giardias may produce hydrogen under anaerobic conditions (Lindmark and Müller, 1973; Reeves, 1984; Brown *et al.*, 1998; Müller, 1998; Lloyd and Harris, 2002). Third, short *fe-hydrogenase* genes of *E. histolytica* and *S. barkhanus* appear to share a common ancestor, even though these two protists are not closely related to each other in phylogenies drawn with rRNA or protein sequences (Sogin and Silberman, 1998; Horner *et al.*, 2000; Bapteste *et al.*, 2002). The latter result suggested the possibility of lateral transfer of *fe-hydrogenase* genes between these protists. Recent phylogenetic studies suggest that numerous genes encoding fermentation enzymes and other proteins of *E. histolytica*, *G. lamblia*, and *T. vaginalis* may have been laterally transferred from prokaryotes (Rosenthal *et al.*, 1997; Doolittle, 1998, 1999; de Koning *et al.*, 2000; Field *et al.*, 2000; Nixon *et al.*, 2002).

With the goal of understanding better the biochemistry and evolution of Fe-hydrogenases of entamoebas and diplomonads, we performed the following studies. (1) The *E. histolytica* short Fe-hydrogenase 1 was expressed as a glutathione-S-transferase (GST) fusion-protein in *E. coli*, and its activity was measured *in vitro*. (2) The *G. lamblia fe-hydrogenase* gene was identified from shotgun genomic sequences, and mRNAs encoding short Fe-hydrogenases were detected in cultured giardias and entamoebas. (3) An *E. histolytica* gene encoding a long Fe-hydrogenase 2 was identified from genomic sequences and compared with other long Fe-hydrogenases. (4) Phylogenetic analyses were repeated with the addition of Fe-hydrogenases of *G. lamblia*, *E. histolytica*, the green alga *Chlamydomonas reinhardtii*, and the eubacterium *Megasphaera elsdenii*.

Materials and Methods

Cloning of the G. lamblia fe-hydrogenase *and* E. histolytica *genes and identification of mRNAs encoding Fe-hydrogenase from cultured entamoebas and giardias.* An *E. histolytica* EST (GenBank AB002772), which encodes the N-terminus of a putative short Fe-hydrogenase 1, was identified from GenBank using BLASTP and an amebic 2[4Fe-4S] ferredoxin sequence (Altschul *et al.*, 1997; Nixon *et al.*, 2002). The 3′ end of the *E. histolytica fe-hydrogenase 1* gene was isolated using 3′ RACE (FirstChoice RLM-RACE kit, Ambion Inc., Austin, Texas), and the entire entamoebic *fe-hydrogenase 1* gene was cloned, sequenced on both strands, and deposited in GenBank under accession number AAG09783. A second *Entamoeba fe-hydrogenase* gene, which encodes a long Fe-hydrogenase, was identified from assemblies of *E. histolytica* genome sequences at The Institute for Genomic Research. The *E. histolytica fe-hydroge-*

nase 2 gene was deposited in GenBank under accession number AY172963.

A shotgun clone from the *G. lamblia* genome sequencing project, which contained the 5′ end of a putative *fe-hydrogenase* gene, was used to identify the entire *fe-hydrogenase* gene from a *G. lamblia* genomic DNA library made in Lambda Zap (McArthur *et al.*, 2000). The *G. lamblia fe-hydrogenase* gene was sequenced on both strands and deposited in GenBank under accession number AAK28337. The N-termini of predicted entamoebic and giardial Fe-hydrogenases were examined with MITOP to determine whether organelle-targeting sequences might be present (Claros and Vincens, 1996).

Total RNA was prepared by lysing cultured entamoebas and giardias in a guanidinium isothiocyanate solution and by centrifuging the lysate through a cesium chloride gradient (Choczynski and Sacchi, 1987). Reverse-transcriptase and polymerase chain reaction (RT-PCR) were performed with these RNAs and with primers specific for the *E. histolytica* and *G. lamblia* genes encoding short Fe-hydrogenase, malic enzyme, alcohol dehydrogenase E (ADHE), and ferredoxin 1 (entamoebas only) (Rosenthal *et al.*, 1997; Nixon *et al.*, 2002). For negative controls, PCR was performed without RT, and RT-PCR products were identified on agarose gels.

Expression of a recombinant short E. histolytica *Fe-hydrogenase and measurement of its activity.* A recombinant glutathione-S-transferase (GST) fusion-protein containing a short Fe-hydrogenase at its C-terminus was made by cloning the *E. histolytica* Fe-hydrogenase 1 coding region into the pGEX-6T vector (Smith and Johnson, 1988). The GST-Fe-hydrogenase 1 construct was transfected into *Escherichia coli* strain BL21, which was grown anaerobically and induced with isopropyl β-D-thiogalactopyranoside (IPTG). Bacteria were lysed by freezing and thawing, and the hydrogenase activities of the supernatant and of purified GST-Fe-hydrogenase 1 were determined. As a negative control, the activity of bacterial lysate expressing a GST-chitinase fusion protein was measured. In a septum-sealed cuvette, Fe-hydrogenase 1 activity was examined by incubation with 10 m*M* methyl viologen in 50 m*M* Tris-HCl (pH 8.0) and 5 m*M* dithioreitol, which had been bubbled with hydrogen for 15 min (Payne *et al.*, 1993). Reduction of methyl viologen was monitored at 600 nm. A negative control was bubbled with nitrogen instead of hydrogen.

We also measured Fe-hydrogenase activity from lysates of cultures of non-transformed *T. vaginalis* and from *E. histolytica* that had been transformed with a plasmid containing the entamoebic *fe-hydrogenase 1* gene under an *actin* gene promoter (Ghosh *et al.*, 2000). This construct was previously used to demonstrate the cytosolic location of the entamoebic Fe-hydrogenase 1.

Phylogenetic analyses. Amino acid sequences homologous to the *G. lamblia* and *E. histolytica* Fe-hydrogenases, which were identified in the nonredundant and unfinished microbial databases of GenBank using BLASTP and TBLASTN, respectively, were aligned using CLUSTALW (Thompson *et al.*, 1994; Altschul *et al.*, 1997). The alignment was adjusted manually using the SEQLAB program (Genetics Computer Group, Madison, Wisconsin). Regions that could not be unambiguously aligned were excluded, leaving 351 aligned amino acid positions for use in phylogenetic analyses. Phylogenetic analyses were performed using distance and parsimony methods by the computer programs TREE-PUZZLE (Strimmer and von Haeseler, 1996) and PHYLIP (Felsenstein, 1989). Pairwise distances were computed using TREE-PUZZLE under the Dayhoff model (Dayhoff *et al.*, 1978), with the inclusion of observed amino acid frequencies, estimated proportion of invariant sites, and estimation of among-site variation for the remaining sites according to a gamma distribution (four discrete categories). The optimal tree was inferred using the Fitch-Margoliash algorithm (Fitch and Margoliash, 1967), using the Fitch program, with global rearrangements and 100 random-addition replicates. Bootstrap values were obtained, using the 100 resampled datasets, under the same model using the PUZZLEBOOT program (available at http://www.tree-puzzle.de). The optimal tree under parsimony and related bootstrap values were determined using PHYLIP's PROTPARS program for parsimony.

Results and Discussion

A recombinant short Fe-hydrogenase 1 of E. histolytica *is active.* The amino-terminus of the *E. histolytica* Fe-hydrogenase 1 did not contain an organelle-targeting sequence (Claros and Vincens, 1996; Horner *et al.*, 2000). Indeed, an epitope-tagged Fe-hydrogenase 1 is present within the cytosol of transfected *E. histolytica* (Ghosh *et al.*, 2000). The activity of the entamoebic Fe-hydrogenase 1 was measured in lysates of *E. coli*, which were expressing a GST-entamoebic Fe-hydrogenase 1 fusion-enzyme (Table 1) (Smith and Johnson, 1988). The activity of the recombinant entamoebic Fe-hydrogenase 1 was present when hydrogen was bubbled into the medium but absent when nitrogen (negative control) was bubbled into the medium (Payne *et al.*, 1993). There was no Fe-hydrogenase activity in control *E. coli*, which were overexpressing a GST-chitinase fusion-protein. Recombinant entamoebic Fe-hydrogenase 1, which was purified on glutathione-agarose beads, had a decreased specific activity (data not shown). This was likely caused by exposure to oxygen during the purification procedure that probably inactivated the Fe-hydrogenase 1 iron-sulfur centers (Cammark, 1992; Payne *et al.*, 1993; Horner *et al.*, 2000). The specific activity of the GST-Fe-hydrogenase 1 fusion enzyme was about 10 times that of the native entamoebic Fe-hydrogenase 1 overexpressed in transfected *E. histolytica* (Table 1). Interestingly, the specific activity of

Table 1

Activities of Entamoeba histolytica *and* Trichomonas vaginalis *Fe-hydrogenases*

Sample	Hydrogenase activity (nmol/min/mg of protein)[a]
Bacteria transformed with *E. histolytica* Fe-hydrogenase	36 (2)
Transfected *E. histolytica* with Fe-hydrogenase	3.5 ± 0.7 (3)
Trichomonas vaginalis	167 ± 32 (9)
T. vaginalis + 0.47 mg *E. histolytica* lysate[b]	114 (2)

[a] Averages +/− standard deviations, where possible. Number of determinations in parentheses.

[b] Calculated K_i = 0.56 mg (amount of *E. histolytica* lysate in mg of protein to cause 50% reduction of *T. vaginalis* hydrogenase activity).

the *T. vaginalis* Fe-hydrogenase was greater than that of the recombinant entamoebic GST-entamoebic Fe-hydrogenase and was inhibited by a lysate of non-transfected entamoebas (Table 1). This may explain why it was difficult to detect Fe-hydrogenase activity in lysates of nontransfected entamoebas, even though Fe-hydrogenase 1 mRNAs were identified from them by RT-PCR (next section).

Cultured entamoebas and giardias express mRNAs encoding short Fe-hydrogenases. We isolated an *fe-hydrogenase* gene of *G. lamblia*, because we have frequently compared the fermentation enzymes of this diplomonad with those of *E. histolytica* (Rosenthal *et al.*, 1997; Field *et al.*, 2000; Nixon *et al.*, 2002). A search of the contigs predicted from the *G. lamblia* shogun sequences suggested that this gene, which predicts a short Fe-hydrogenase, is the only hydrogenase gene present within the giardial genome. Like the entamoebic Fe-hydrogenase 1, the predicted giardial Fe-hydrogenase lacked an N-terminal organelle-targeting sequence and had two ferredoxin-like iron-sulfur centers and a hydrogenase iron-sulfur center like those present in the short Fe-hydrogenases of *T. vaginalis*, *Desulfovibrio* sp., and *Clostridia* sp. (Cammark, 1992; Thompson *et al.*, 1994; Bui and Johnson, 1996; Horner *et al.*, 1996; Nicolet *et al.*, 1999). RT-PCR showed that cultured entamoebas and giardias contain mRNAs, which encode short Fe-hydrogenases (Fig. 1A, B). Negative controls without RT showed that the RT-PCR was not amplifying DNA from the extracts of cultured entamoebas and giardias. Because the giardial contigs predicted only one Fe-hydrogenase, which is expressed, it is likely that the hydrogenase activity recently detected in cultures of giardias derives from this enzyme (Lloyd and Harris, 2002). In contrast, entamoebas appear to have a second long hydrogenase (see next section), so if entamoebic hydrogenase activity is present, it might derive from one or more enzymes. These results suggest the possibility that entamoebas and giardias use protons as electron acceptors

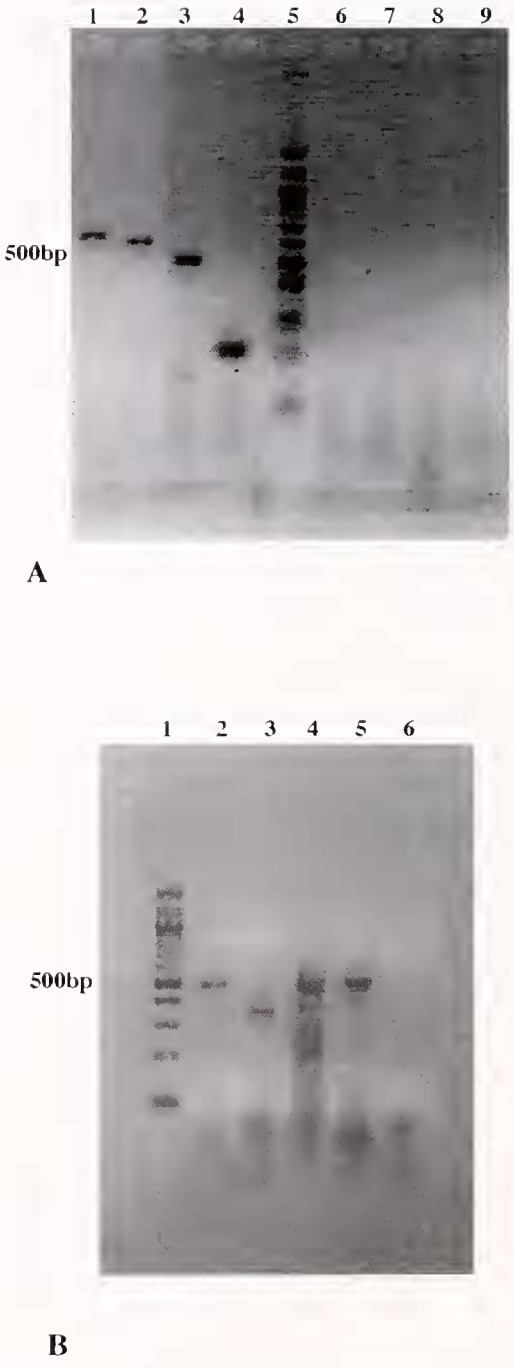

Figure 1. Agarose gels of ethidium-stained RT-PCR products from entamoebic and giardial mRNAs. Images are reversed for clarity of reproduction. (A) RT-PCR of amoebic mRNAs encoding malic enzyme (lane 1), Fe-hydrogenase (lane 2), alcohol dehydrogenase E (lane 3), and ferredoxin (lane 4). Size markers are shown in lane 5. Lanes 6–9 are the negative controls for malic enzyme, hydrogenase, ADHE, and ferredoxin, respectively. (B) RT-PCR of giardial mRNAs encoding ADHE (lane 3), Fe-hydrogenase (lane 4), and malic enzyme (lane 5). A negative control (no RT) for Fe-hydrogenase is shown in lane 6. A positive control for Fe-hydrogenase, using *Giardia lamblia* WB strain DNA, is shown in lane 2. Size markers are shown in lane 1.

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Eh2  MSTQLTPLRNKIISEVVKCFKSGRFIEDIDKLPTILTDGDGWKPTSKFVHSREQEEGIYR
Td           IKREILVRIAKLQFEGKLQEGVHYIPREMVPRN.STPI.RCCIFHDR..EIMR
Bf           VRHKLLAKLVNLWKENKLTNEIDRLPIELSPRR.SRPLGRCCIHKER..AVYK

Eh2  EKVLSVLGF.VDGEYDDITPLHVYAQKALERT.SLHEPVFGISQKGCNKCHFNGYFVTQA
Td   HRVIARLGCSLENYDEEKT.LAQFAKEALERE.KPTWPMLTVLDEACNACVKSKYMITNA
Bf   YKLFPLLGFDMTDETDELTSLSEYARQALERKNKQKENILCVIDEACSSCVQVNYEVTNL
                                                      *  *

Eh2  CEGCTSRPCSVNCPKKCISFGEDGRAVINQNNCIKCGRCYKFCPYGAIISKSVPCVKACP
Td   CQACVARPCMMNCPKTAIAIS.GGRARIDEEKCINCGICLKNCPYHAVIKIPVPCEEACP
Bf   CRGCVARSCYMNCPKDAIRFRKNGQAKIDHDACISCGKCHQSCPYHAIVFIPVPCEEACP
     *  *    *   *                        x  x  x   x           *   *

Eh2  CGAMLDSPEGVKTIDFEKCINCGGCMRACPFGAILPRSNLIDVLK.ILPTKKVVACPAPS
Td   VGAISKDENGKERIDYHKCIFCGNCMRECPFGAMMDKGQIVDVIKHLMSGKKVSALYAPA
Bf   VKAISKDENGIEHIDESKCIYCGKCLNACPFGAIFEISQAFDVLEGIRSGEKMIAIPAPS
                       x  x  x   x

Eh2  IAAHFGKYDLALVSGGLIQVGFTSVEDVSYGADLCALNEAKEFEERIVKNKKDFMTTSCC
Td   VAAQF.KAVPGQLESALKKAGFNKVWEVAIGADITADREASEFEERMEHGHI.LMTTSCC
Bf   ILGQF.NTSIEAVYGALRQMGFADVVEVAQGAMDTVSHEAAELKEKLEEGQP.FMTTSCC
                                                          o

Eh2  PAYINAINKHMPELKENVSHTPTPMHFATQAVKDRDQETVTVFIGPCNAKRWETLQDSTT
Td   PAYVRAVKKHVPALVPCISDTRSPMHYTAELAKKEDPDCVTVFIGPCLAKRREGLEDEFV
Bf   PSYIELVNKHIPGMKPYVSSTGSPMYYAARIAKERHPDAKIVFIGPCVAKRKEARRDECV
                                                    o

Eh2  DYCLTFDEIFGLFEGSGIDLSKVQPYTFVDKAHKEGKIFAVSGGVASAVASLLPKEVPDG
Td   DYVLSIEELGALLTAKEIDISKEEALPGKITPTSSGRGFAASGGVAEAVRVRL.KKPEN.
Bf   DYILTFEEMASIFEGLDIQLEQTQPFSVLYTSVREAHGFAQAGGVMGAIKAYLGEEAKK.

Eh2  VIKPTIIDGFSQENFKRLKNFKKNI.........TGNLVEVMVCEGGCAYGPGCPGLNTP
Td   .LRPVLINGLNKEGMKQLASYGKIQSGELPHDSSTPNLVEVMSCEGGCIGGP
BF   .FSAIQVSDLNKKNIGLLRAAAKTG.......KAQGQFIEVMACEGGCISGP
                                                    o   o

Eh2  ATSAKIKIAVDKMEAHPEGRWVGLPNSQIKPIKVEN 504
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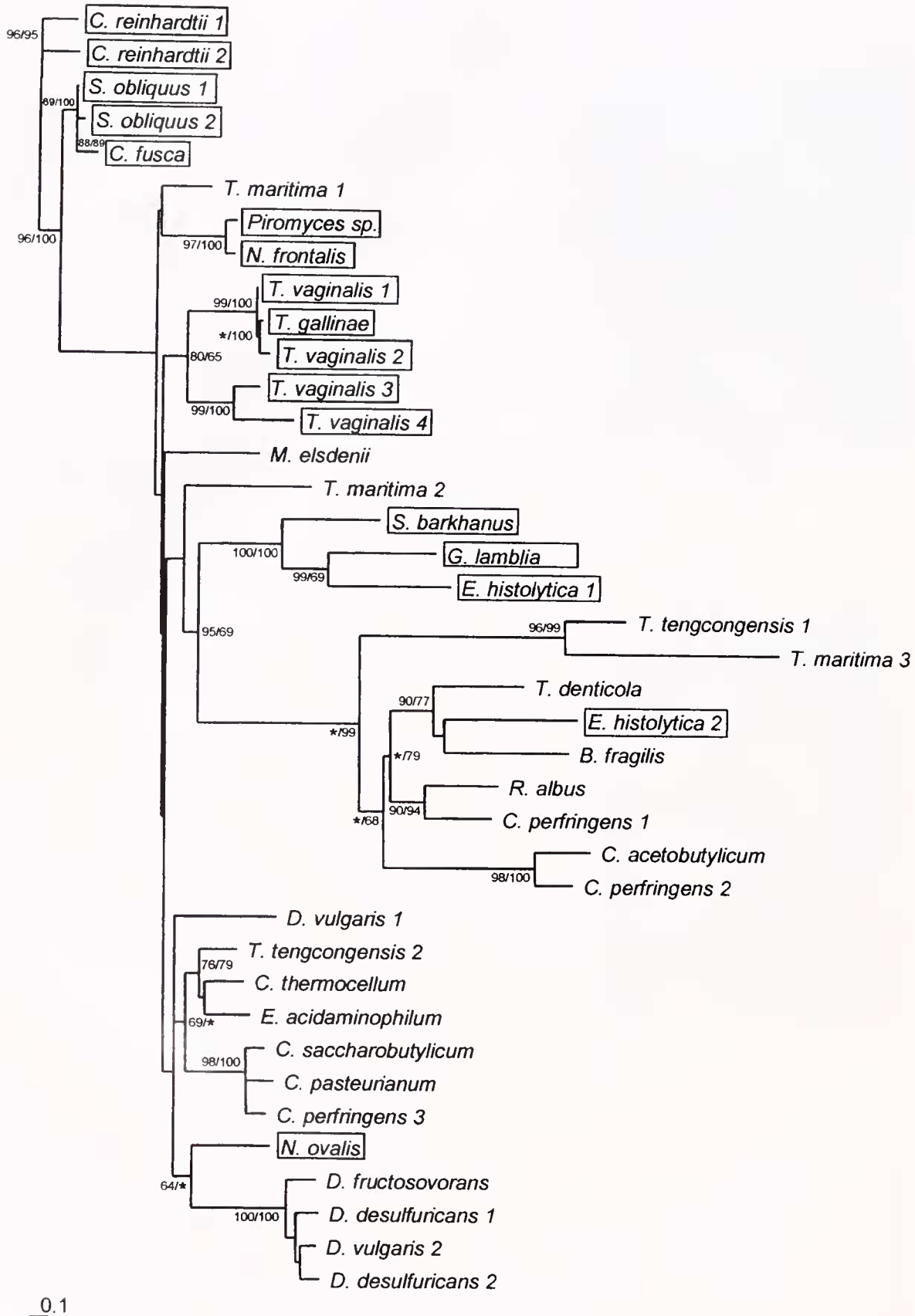
Figure 2. Alignment of the predicted *Entamoeba histolytica* long Fe-hydrogenase 2 (Eh2) with predicted long Fe-hydrogenases of *Treponema denticola* (Td) and *Bacteroides fragilis* (Bf). Conserved Cys residues, which are shaded, include those that coordinate putative [4Fe-4S] iron-sulfur centers (marked with x's) and those that coordinate putative hydrogenase iron-sulfur centers (marked with o's). Other conserved Cys residues, which may be involved in coordinating iron-sulfur centers, are marked with asterisks. Amino acids at the beginning and end of the conserved Fe-hydrogenase domain are underlined.

when the organisms are growing under strictly anaerobic conditions in the bowel lumen (Brown *et al.*, 1998; Huston and Petri, 2001; Lloyd and Harris, 2002).

E. histolytica *has a* hydrogenase 2 *gene encoding a long Fe-hydrogenase.* The assemblies of the shotgun sequences of the *E. histolytica* genome predicted a long Fe-hydrogenase 2 (Fig. 2) in addition to the short Fe-hydrogenase 1. The entamoebic Fe-hydrogenase 2 was 504 amino acids long and had an N-terminal sequenee, which included positively charged Lys and Arg that are often present at the N-termini of organellar proteins (Claros and Vincens, 1996). In addition, the N-terminus of Fe-hydrogenase 2 contained Ser and Leu residues, which are present at the N-termini of crypton and hydrogenosomal proteins (Bui *et al.*, 1996; Mai *et al.*, 1999). However, in the absence of experimental evidence, we cannot be sure that the entamoebic long Fe-hydrogenase is targeted to the crypton.

The entamoebic Fe-hydrogenase 2 was much more similar (>38% amino acid identities) to predicted long Fe-hydrogenases of *Bacteroides fragilis* and *Treponema denticola* than to short Fe-hydrogenases of entamoebas, giardias, trichomonads, and other anaerobic bacteria (<28% amino acid identities; Fig. 2). The entamoebic Fe-hydrogenase 2 and the predicted long Fe-hydrogenases of *B. fragilis*


C. reinhardtii 1
C. reinhardtii 2
S. obliquus 1
S. obliquus 2
C. fusca
T. maritima 1
Piromyces sp.
N. frontalis
T. vaginalis 1
T. gallinae
T. vaginalis 2
T. vaginalis 3
T. vaginalis 4
M. elsdenii
T. maritima 2
S. barkhanus
G. lamblia
E. histolytica 1
T. tengcongensis 1
T. maritima 3
T. denticola
E. histolytica 2
B. fragilis
R. albus
C. perfringens 1
C. acetobutylicum
C. perfringens 2
D. vulgaris 1
T. tengcongensis 2
C. thermocellum
E. acidaminophilum
C. saccharobutylicum
C. pasteurianum
C. perfringens 3
N. ovalis
D. fructosovorans
D. desulfuricans 1
D. vulgaris 2
D. desulfuricans 2
96/95
69/100
88/89
96/100
97/100
99/100
*/100
80/65
99/100
100/100
99/69
95/69
96/99
90/77
*/99
*/79
90/94
*/68
98/100
76/79
69/*
98/100
64/*
100/100
0.1

and *T. denticola* each contained Cys residues that likely coordinate two ferredoxin-like [4Fe-4S] iron-sulfur centers (marked with x's in Fig. 2) and hydrogenase iron-sulfur centers (marked with o's), which have previously been identified in structures of short and long Fe-hydrogenases (Peters *et al.*, 1998; Nicolet *et al.*, 1999). In addition, the predicted entamoebic Fe-hydrogenase 2 had eight other N-terminal Cys residues, which aligned with those of the bacteroides and treponema long Fe-hydrogenases (marked with asterisks). Although these Cys residues probably coordinate other iron-sulfur centers, they remain unidentified, because they do not align with the N-terminal iron-sulfur centers of the long Fe-hydrogenase of *C. pasteurianum*, which has been crystallized (Peters *et al.*, 1998).

The entamoebic and giardial short fe-hydrogenase 1 *genes appear to share a common ancestry, while the entamoebic long* fe-hydrogenase 2 *gene appears to have been laterally transferred from a prokaryote.* Phylogenetic trees of Fe-hydrogenases from eubacteria and eukaryotes are star-shaped and contain few basal nodes that are strongly supported (Fig. 3). This result suggests that the Fe-hydrogenases are widely divergent and that little phylogenetic signal remains. For example, Fe-hydrogenases of closely related eukaryotes—either trichomonads, green algae (*Chlamydomonas reinhardtii*, *Scenedesmus obliquus*, and *Chlorella fusca*), or chytrid fungi (*Piromyces* sp. and *Neocallimastix frontalis*)—each grouped together, but Fe-hydrogenases of unrelated eukaryotes did not group together. In particular, our analysis does not support recent conclusions that hydrogenases of trichomonads are monophyletic with those of chytrid fungi (Voncken *et al.*, 2002) or with those of *E. histolytica* and *S. barkhanus* (Horner *et al.*, 2000).

The short *fe-hydrogenase* genes of *G. lamblia*, *S. barkhanus*, and *E. histolytica* appear to share a most recent common ancestry, although a particular bacterial donor was not identified. Remarkably, the short Fe-hydrogenase of *G. lamblia* was more similar to that of *E. histolytica* than to that of *S. barkhanus*. Because *G. lamblia* and *S. barkhanus* are diplomonads, which share a recent common ancestor in phylogenetic trees of rRNA and proteins (Sogin and Silberman, 1998), a possible explanation of these results is that the *E. histolytica fe-hydrogenase* gene was laterally transferred from a diplomonad (Rosenthal *et al.*, 1997; Doolittle, 1998, 1999; Müller, 1998; de Koning *et al.*, 2000; Field *et al.*, 2000; Nixon *et al.*, 2002). This lateral gene transfer would not have occurred recently, because the Fe-hydrogenases of entamoebas and giardias showed only a 40% amino acid identity with each other, and each *fe-hydrogenase* gene has the codon usage of its host. Alternatively, the diplomonad-*E. histolytica* sub-clade could be incorrectly rooted by the long branch connecting it to the remainder of the tree.

The common ancestry of genes encoding the *E. histolytica* long Fe-hydrogenase 2 and those of *B. fragilis* and *T. denticola* is strongly supported. This appears then to be an example of lateral gene transfer, as *Entamoeba* is not a close relative of either of these eubacteria (Rosenthal *et al.*, 1997; Doolittle, 1998, 1999; Müller, 1998; de Koning *et al.*, 2000; Field *et al.*, 2000; Nixon *et al.*, 2002). There was weak support for the pairing of Fe-hydrogenases of the ciliate *N. ovalis* and *Desulfovibrio* sp., as has been previously noted (Horner *et al.*, 2000; Voncken *et al.*, 2002). This suggests that the ciliate hydrogenase was derived by lateral gene transfer, but does not prove it.

Conclusions

This is the first time that an Fe-hydrogenase from a protist has been expressed as a GST-fusion protein in bacteria. This is also the first time that an *fe-hydrogenase* gene (encoding the long hydrogenase of entamoebas) has been inferred to have been laterally transferred from a bacterium, although numerous genes encoding fermentation enzymes (*e.g.*, alcohol dehydrogenases, malic enzyme, and acetyl-CoA syn-

Figure 3. Phylogenetic relationships of Fe-hydrogenases, inferred using a distance matrix generated by the Dayhoff+1+Γ model and the Fitch-Margoliash algorithm. Bootstrap values obtained using PUZZLEBOOT and PROTPARS, respectively, are shown at the relevant nodes. Bootstrap values below 50% are marked with an asterisk if the other bootstrap value is >50%. If both bootstrap values are below 50%, neither is marked. The scale bar indicates estimated sequence divergence per unit branch length. Sequences from eukaryotes, which are boxed, were from *Chlamydomonas reinhardtii* 1 and 2 [accession # 16945126 and 18026272]; *Chlorella fusca* [21732235]; *Entamoeba histolytica* 1 and 2 [9963974 and AY172963]; *Giardia lamblia* [13506793]; *Neocallimastix frontalis* [19547863]; *Nyctotherus ovalis* [4034791]; *Piromyces sp.* [19548180]; *Scenedesmus obliquus* 1 and 2 [12581498 and 13311187]; *Spironucleus barkhanus* [11127703]; *Trichomonas gallinae* [19548182]; *Trichomonas vaginalis* 1, 2, 3, and 4 [19547859, 11127701, 1171117, and 1345094]. Eubacterial sequences were from *Bacteroides fragilis* [unfinished microbial database]; *Clostridium acetobutylicum* [15896476]; *Clostridium pasteurianum* [557064]; *Clostridium perfringens* 1, 2, and 3 [18311557, 18309258, and 18311328]; *Clostridium saccharobutylicum* [488597]; *Clostridium thermocellum* [4927278]; *Desulfovibrio fructosovorans* [1914864]; *Desulfovibrio desulfuricans* 1 and 2 [4930044 and 13022069]; *Desulfovibrio vulgaris* 1 and 2 [97381 and 66319, respectively]; *Eubacterium acidominophilum* [14250935]; *Megasphaera elsdenii* [6650985]; *Ruminococcus albus* [unfinished microbial database]; *Thermoanaerobacter tengcongensis* 1 and 2 [20807184 and 20515894]; *Thermotoga maritima* 1, 2, and 3 [15644177, 7433127, and 4981985]; and *Treponema denticola* [unfinished microbial database].

thases) appear to have been laterally transferred from prokaryotes to amoebas and giardias (Rosenthal *et al.*, 1997; Field *et al.*, 2000; Nixon *et al.*, 2002). Although the evidence is weak, this may also be the first time that a gene (encoding the short hydrogenase of entamoebas) has been inferred to have been laterally transferred from another protist. Because the hypothesized lateral gene transfer would probably have occurred after the acquisition of the *fe-hydrogenase* gene by the diplomonad lineage, this particular result does not disprove the hydrogen hypothesis (Martin and Müller, 1998). However, the failure to demonstrate that the eukaryotic Fe-hydrogenases share a common ancestry, or to identify an α-proteobacterial donor for these eukaryotic *fe-hydrogenase* genes (Horner *et al.*, 2000), dampens our enthusiasm for the hydrogen hypothesis. These results suggest that the mitochondrial endosymbiont was selected for a property other than hydrogen production (*e.g.*, its ability to consume oxygen) (Andersson and Kurland, 1999) and that the presence of Fe-hydrogenases and other fermentation enzymes of microaerophilic eukaryotes may reflect a secondary adaptation to their anaerobic environment (Rosenthal *et al.*, 1997; Doolittle, 1998, 1999; de Koning *et al.*, 2000; Field *et al.*, 2000; Lloyd and Harris, 2002; Nixon *et al.*, 2002).

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